Mutagenesis vol. 22 no. 5 pp. 305–310, 2007
Advance Access Publication 4 June 2007
doi:10.1093/mutage/gem017

Genetic polymorphisms of manganese-superoxide dismutase and glutathione-*S*-transferase in chronic alcoholic pancreatitis

Christoph H. Österreicher[1], Jürgen Schultheiss[2], Markus Wehler[3], Nils Homann[4], Claus Hellerbrand[5], Beat Künzli[6], Helmut Friess[6], Helmut K. Seitz[7] and Felix Stickel[8,*]

[1]Department of Medicine, Columbia University, NY, USA, [2]Interdisciplinary Center of Clinical Research, University of Erlangen-Nuremberg, Erlangen, Germany, [3]Department of Medicine I, University of Erlangen-Nuremberg, Erlangen, Germany, [4]Department of Medicine I, University of Lübeck, Lübeck, Germany, [5]Department of Medicine I, University of Regensburg, Regensburg, Germany, [6]Department of General Surgery, University of Heidelberg, Heidelberg, Germany, [7]Department of Medicine, Salem Medical Center, University of Heidelberg, Heidelberg, Germany and [8]Institute of Clinical Pharmacology, University of Berne, Switzerland

Chronic alcohol consumption is a major risk factor for the development of chronic pancreatitis. However, chronic pancreatitis occurs only in a minority of heavy drinkers. This variability may be due to yet unidentified genetic factors. Several enzymes involved in the degradation of reactive oxidants and xenobiotics, such as glutathione-*S*-transferase P1 (GSTP1) and manganese–superoxide dismutase (MnSOD) reveal functional polymorphisms that affect the antioxidative capacity and may therefore modulate the development of chronic pancreatitis and long-term complications like endocrine and exocrine pancreatic insufficiency. Two functional polymorphisms of the MnSOD and the GSTP1 gene were assessed by polymerase chain reaction and restriction fragment length polymorphism in 165 patients with chronic alcoholic pancreatitis, 140 alcoholics without evidence of pancreatic disease and 160 healthy control subjects. The distribution of GSTP1 and MnSOD genotypes were in Hardy–Weinberg equilibrium in the total cohort. Genotype and allele frequencies for both genes were not statistically different between the three groups. Although genotype MnSOD Ala/Val was seemingly associated with the presence of exocrine pancreatic insufficiency, this subgroup was too small and the association statistically underpowered. None of the tested genotypes affected the development of endocrine pancreatic insufficiency. Polymorphisms of MnSOD and GSTP1 are not associated with chronic alcoholic pancreatitis. The present data emphasize the need for stringently designed candidate gene association studies with well-characterized cases and controls and sufficient statistical power to exclude chance observations.

Introduction

Chronic pancreatitis is a progressive inflammatory disorder that eventually leads to exocrine and/or endocrine insufficiency. Long-term alcohol consumption is the predominant cause for the development of chronic pancreatitis which, however, develops only in about 5–10% of heavy drinkers (1). Compelling evidence for an important genetic background in the development of chronic pancreatitis derives from the detection of mutations in the cationic trypsinogen (2), the pancreatic serine protease inhibitor Kazal type 1 (3) and the cystic fibrosis gene (4). However, most human data point against a decisive role of the above mentioned mutations in the pathophysiology of chronic alcoholic pancreatitis (CAP), so, other genetic modifiers are likely responsible. In this regard, candidate gene association studies have investigated the relationship between the development of CAP and single-nucleotide polymorphisms (SNPs) of genes that play a role in the pathophysiology of pancreatic injury (5–8).

Oxidative stress plays an important role in the evolution of CAP since reactive oxygen species (ROS) generated during oxidative alcohol degradation lead to cellular and subcellular damage (9–11). Herewith, variants of genes that code for antioxidant enzymes, such as glutathione-*S*-transferases (GSTs) and manganese–superoxide dismutase (MnSOD), could modulate the susceptibility to develop CAP.

GSTs are a family of four isoenzymes (GSTA, GSTM, GSTT and GSTP) that inactivate ROS and xenobiotics through conjugation with glutathione (GSH) (12). The isoenzyme GSTP1 is expressed in biliary epithelial and pancreatic stellate cells, and its gene reveals a polymorphism which leads to an amino acid substitution (Ile → Val) at codon 105 resulting in a 3-fold decreased capability of GSTP1 to detoxify 1-chloro-2,4-dinitrobenzene (13) and to deactivate 4-hydroxynonenal, a highly reactive product of lipid peroxidation (14). We have recently identified genotype GSTP1 Val/Val as a genetic risk factor for the development of liver cirrhosis in patients with hereditary hemochromatosis, a disease in which oxidative stress is the pivotal trigger of tissue injury (15).

Mitochondria-derived ROS are detoxified to hydrogen peroxide and water by the successive action of MnSOD and GSH peroxidase, respectively (16). MnSOD is synthesized with a cleavable target sequence that enables its transport into mitochondria (17). A dimorphism (C → T) at codon 16 leads to either alanine (Ala) or valine (Val) at amino acid position −9 of the target sequence resulting in its enhanced translocation into mitochondria and a 40% higher concentration of active MnSOD in case of the Ala sequence (18,19). Previous human studies focused on the role of MnSOD variants in the evolution of alcoholic liver disease but yielded controversial results with a positive association in the initial report (20) and negative findings in the later, and larger, study (21).

In spite of the crucial role of oxidative stress in the evolution of CAP, only few small studies investigated the role of functional polymorphisms of genes encoding GST and MnSOD isoenzymes (22–25) with inconclusive results. Therefore, in the present study, we investigated whether

*To whom correspondence should be addressed. Tel: +49 31 632 8715; Fax: +49 31 632 4997; Email: felix.stickel@ikp.unibe.ch

genetic polymorphisms of GSTP1 and MnSOD are associated with the occurrence of chronic pancreatitis and its complications in a large cohort of heavy drinkers.

Patients and methods

Patients

The total study cohort comprised 305 alcoholic patients enrolled between 2000 and 2003 and 160 healthy individuals (118 men, 42 women) from hospital and research laboratory staff who served as a reference population with regard to the distribution of MnSOD and GSTP1 genotypes. All included patients were Caucasians of German descent. The present and past daily alcohol intake of all recruited alcoholic subjects was recorded through standardized interrogation during a face-to-face interview. Into the two groups of alcoholics, female and male patients with active or recent heavy alcohol consumption as defined by at least 80 g/day for >10 years were included. Patients were assigned to either group CAP or group of alcoholics without pancreatitis (ALC). Diagnosis of CAP was based on a history of long-lasting heavy alcohol consumption and the presence of chronic pancreatitis as evidenced by histologic criteria and/or a combination of morphologic, functional and clinical findings. A diagnosis of chronic pancreatitis was considered confirmed if a score of 4 or more was achieved using the previously described Mayo Clinic Score (26): 4, typical histological changes; 4, pancreatic calcification; 3, characteristic findings on endoscopic retrograde cholangiopancreatography, and 2, exocrine pancreatic insufficiency (steatorrhea by abnormal quantitative fecal fat excretion >7 g/day or an abnormal secretin–pancreozymin test result). A score of 2 was assigned for attacks of pancreatitis and/or chronic upper abdominal pain, and a score of 1 was assigned for diabetes mellitus (glucose intolerance requiring continuous control by diet or with addition of oral agents or insulin).

The diagnosis of chronic diarrhea due to pancreatic maldigestion was based on the diagnosis of chronic pancreatitis, increased frequency (>3/day) and liquidity of stools, and exocrine pancreatic insufficiency proven by steatorrhea, abnormal secretin–pancreozymin test result or fecal elastase 1 concentration <200 μg/g stool in three different stool samples (27). Other causes of diarrhea had to be excluded and stool samples were lyophilized before determination of elastase 1 to prevent false low results (28). The presence of exocrine pancreatic insufficiency applied to 75 of 165 patients with CAP. Endocrine pancreatic insufficiency was defined by the presence of diabetes mellitus together with decreased starving serum insulin and C-peptide concentrations, and the absence of criteria of the metabolic syndrome which was present in 36 of 165 patients with CAP.

ALC were active heavy drinkers recruited from patients admitted to the hospital for alcohol detoxification. Absence of alcoholic pancreatitis or liver cirrhosis was confirmed by the absence of clinical, biochemical, radiological and/or endoscopic evidence. Subjects included in this group belonged to a subgroup of 174 alcoholic patients previously genotpyed in a study testing alcohol dehydrogenase 1C polymorphisms as a possible genetic risk factor for alcohol-related cancers (29). Of these patients, 34 were excluded for the present study because of clinical signs of infection and/or and accident injuries.

The study protocol was reviewed and approved by the local Ethics Committees of the four participating centers (Erlangen, Heidelberg, Regensburg and Lübeck), and all patients and healthy volunteers gave written informed consent prior to inclusion.

Mutation analysis

For genotyping procedures, genomic DNA was isolated from peripheral blood using the QIAamp blood DNA extraction kit (Qiagen GmbH, Hilden, Germany). Polymerase chain reactions (PCRs) were performed with 100–200 ng of genomic DNA in a 25-μl reaction volume containing 0.2 mmol of each primer, 200 mmol of nucleotides (Peqlab Biotechnologies, Erlangen, Germany) and 2.5 U AmpliTaq Gold polymerase (Qiagen GmbH). All primers were synthesized by MWG Biotech, Ebersberg, Germany.

The GSTP1 codon 105 polymorphism was analyzed as reported (15) using as forward primer 5′-ACC CCA GGG CTC TAT GGG AA-3′ and as reverse primer 5′-TGA GGG CAC AAG AAG CCC CT-3′. After a 'hot start' at 95°C, the conditions were set at 35 cycles at 95°C for 1 min, followed by 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. For restriction fragment length polymorphism analysis, the 176-bp amplification product was digested with BsmA1 (New England Biolabs, Frankfurt, Germany) at 55°C for 12 h giving rise to either an undigested 176-bp fragment, representing the Ile^{105}-encoding allele, or two fragments of 91 and 85 bp, respectively, representing the Val^{105}-encoding allele.

The PCR procedure to determine MnSOD genotypes was carried out as previously described (15) by using the following primers (MWG Biotech): forward primer 5′-CAG CCC AGC CTG CTG AGA CGG-3′ and reverse primer 5′-CTT GGC CAA CGC CTC CTG GTA CTT-3′. The reaction volumes and the cycling conditions were the same as for the GSTP1 genotyping procedure. The PCR generated an amplicon of 267 bp that was subjected to digestion with endonuclease BsaW1 (New England Biolabs) for 12 h at 60°C. This restriction enzyme only cuts the MnSOD amplification product into two fragments of 183 and 84 bp, respectively, when a thymine is present at position 1183 of the MnSOD gene (Val construct). The digestion products were analyzed on 2.5% agarose gel as detailed for GSTP1. In order to assure the reliability of the genotyping methods, 25% of DNA samples was genotyped twice by two examiners (F.S. and J.S.).

Statistical analysis

Student's *t*-test was conducted to test for differences in means between groups. Skewed data as assessed by the Kolmogorov–Smirnov test were summarized by median and range. Comparison of these groups or groups with unequal variances, as assessed by Levine's test, was done by applying the Mann–Whitney *U*-test. Categorical data were summarized by frequencies and analyzed by a chi-square test. Binary logistic regression analysis with forward and backward stepwise inclusion of variables was calculated to assess the impact of laboratory and clinical data and of GSTP1 and MnSOD genotypes on the presence of alcoholic pancreatitis and endocrine and exocrine pancreatic insufficiency. *P*-values of <0.05 (two-tailed) were considered significant. A commercially available software program SPSS10.0 (SPSS Inc., Chicago, IL, USA) was used for all calculations. Testing for deviation from Hardy–Weinberg equilibrium was done by the use of the following homepage: http://ihg.gsf.de/cgi-bin/hw/hwa1.pl.

Sample size–power calculation was done by the use of the following homepage: http://www.meduniwien.ac.at/medstat/research/samplesize/ssize.html.

Results

A total number of 465 individuals were recruited for this study and genotyped for genetic variants of MnSOD and GSTP1. Table I summarizes the demographic characteristics of the two patient groups. Patients with CAP were predominantly male and significantly older than ALC. The proportion of smokers was generally high in both groups but even larger in those with CAP. Due to the large recall bias, only present alcohol consumption was quantified and expressed in grams per day, whereas past alcohol consumption was only estimated in terms of length of consumption and recorded in years of consumption. Only patients with an alcohol history of at least 10 years were considered eligible for inclusion. Alcoholics without chronic pancreatitis drank significantly more alcohol than patients with CAP. Healthy control subjects drank little or no alcohol at all (<20 g), but 23% of all included subjects were smokers.

Table II shows the genotype and allele frequencies of both studied genes in patients with CAP, chronic alcoholics and healthy controls. There was no difference in the frequency of genotypes and alleles between these three groups. In particular, genotype distribution in healthy controls did not differ from chronic alcoholics and patients with CAP. The distribution of GSTP1 and MnSOD variants were in Hardy–Weinberg equilibrium in the total cohort.

In order to account for the significant differences in the demographic characteristics of the two groups of alcoholics with regard to gender and age, as well as for alcohol and tobacco consumption, a logistic regression analysis was conducted. Results are presented in Table III. Herein, age, gender, alcohol and tobacco consumption were identified as independent risk factors for the development of alcoholic chronic pancreatitis. Since none of the genotypes were found associated with chronic pancreatitis as assessed by chi-square test, they were not included in the logistic model.

In a subgroup analysis of patients with CAP who developed exocrine pancreatic insufficiency, a well-known complication of chronic pancreatitis, patients with exocrine pancreatic insufficiency carried more frequently MnSOD genotype Ala/Val than those without (68.0% versus 46.7%, $P = 0.022$) (Table IV). Age, gender, smoking habits and alcohol consumption did not differ significantly between patients with and without exocrine pancreatic insufficiency, respectively. However, the distribution of MnSOD genotypes in this subgroup of patients was not in Hardy–Weinberg equilibrium ($P = 0.002$).

In patients with chronic pancreatitis, neither GSTP1 nor MnSOD genotypes had an impact on the presence of endocrine pancreatic insufficiency (Table V).

Discussion

Our study is among the largest so far to investigate the impact of SNPs of antioxidant enzymes on the development of chronic pancreatitis in heavy drinkers, and the first to study their potential association with well-established complications of chronic pancreatitis. Neither of the two tested functional genetic polymorphic variants was associated with the chronic pancreatitis in alcoholics. The genotyping results presented in our study were in Hardy–Weinberg equilibrium for both genes which render genotyping errors rather unlikely. Moreover, genotype and allele frequencies in our cohort complied with published data from other studies (20,21,30) and with the reference HapMap database (31).

In previous studies by other researchers, genotype MnSOD Ala/Ala was shown to increase the mitochondrial enzyme activity of MnSOD by 40% compared with the other two genotypes (19). So, patients with genotype MnSOD Ala/Ala should have a higher endogenous antioxidant capacity providing a better resistance towards oxidative stress typically encountered in CAP, whereas carriers with MnSOD Val/Val should be more prone to develop alcohol-related pancreatic injury. This would seem even more important since both manganese and copper/zinc SOD enzymes are induced during the spontaneous development of chronic pancreatitis in male WBN/Kob rats (32). Moreover, transgenic mice over-expressing copper/zinc SOD are more resistant to caerulein-induced acute pancreatitis (33). Also, transgenic mice with increased beta-cell expression of MnSOD and catalase showed a markedly

Table I. Characterization of patients and control subjects

	Total	Alcoholics without chronic pancreatitis ($n = 140$)	Alcoholics with chronic pancreatitis ($n = 165$)	P values
Males, N (%)		101 (72.1)	136 (82.4)	0.032
Age (years)				
Mean ± SD	46.2 ± 10.4	41.9 ± 9.5	49.9 ± 9.7	<0.001
Median (range)	45 (22–82)	41 (22–72)	48 (30–82)	<0.001
Alcohol, g/day				
Mean ± SD	173.6 ± 151.7	251.1 ± 145.3	109.9 ± 125.7	<0.001
Median (range)	120 (0–800)	200 (60–800)	80 (0–800)	<0.001
Smokers, N (%)	246 (80.7)	105 (75.0)	141 (85.5)	0.021
Pancreatic calcifications, N (%)	—	—	76 (46.1)	—
Pancreatic pseudocysts, N (%)	—	—	60 (36.4)	—
Elastase activity (μg/g)	—	—		—
With exocrine pancreatic insufficiency			96.6 ± 51.3	
Without exocrine pancreatic insufficiency			402.1 ± 80.5	
Others, N (%)	—	—	32 (19.4)	—

Others: pancreatic duct stenosis ($n = 5$), pancreas necrosis ($n = 3$), pancreas edema ($n = 1$), gastric/duodenal ulcer ($n = 18$), liver cirrhosis ($n = 4$) and splenic vein thrombosis ($n = 1$).

Table II. Results of genotyping analysis

	Healthy controls (n = 160)	Alcoholics without chronic pancreatitis (n = 140)	Alcoholics with chronic pancreatitis (n = 165)	P value*
GSTP1, *N* (%)				
Ile/Ile	75 (46.9)	54 (38.6)	65 (39.4)	0.647*
Ile/Val	64 (40.0)	74 (52.9)	81 (49.1)	
Val/Val	21 (13.1)	12 (8.6)	19 (11.5)	
Ile	0.67	0.65	0.64	*0.785
Val	0.33	0.35	0.36	
MnSOD, *N* (%)				
Ala/Ala	43 (26.9)	42 (30.0)	40 (24.2)	*0.059
Ala/Val	81 (50.6)	60 (42.9)	93 (56.4)	
Val/Val	36 (22.5)	38 (27.1)	32 (19.4)	
Ala	0.52	0.51	0.52	*0.806
Val	0.48	0.49	0.48	

*Comparing alcoholics with pancreatitis.

Table III. Results of binary logistic regression analysis

Factor	Parameter estimate	SE parameter estimate	Odds ratio (95% CI)	P value
Age	0.090	0.017	*	<0.001
Alcohol	−0.008	0.001	*	<0.001
Male sex	0.967	0.349	2.630 (1.327–5.209)	0.006
Smoking	1.250	0.426	3.489 (1.515–8.035)	0.003

*For the variables age and alcohol no odds ratio was calculated, since both variables were not classified into groups and were entered as continuous variables in the regression model.

Table IV. Association of genotypes and alleles with exocrine pancreatic insufficiency

	Exocrine pancreas insufficiency		P-value
	Absent (n = 90)	Present (n = 75)	
GSTP1, *N* (%)			
Ile/Ile	30 (33.3)	35 (46.7)	0.156
Ile/Val	47 (52.2)	34 (45.3)	
Val/Val	13 (14.4)	6 (8.0)	
Ile	0.59	0.69	0.062
Val	0.41	0.31	
MnSOD, *N* (%)			
Ala/Ala	27 (30.0)	13 (17.3)	0.022
Ala/Val	42 (46.7)	51 (68.0)	
Val/Val	21 (23.3)	11 (14.7)	
Ala	0.53	0.51	0.717
Val	0.47	0.49	

Table V. Association of genotypes and alleles with endocrine pancreatic insufficiency

	Endocrine pancreas insufficiency		P value
	Absent (n = 129)	Present (n = 36)	
GSTP1, *N* (%)			
Ile/Ile	53 (41.1)	12 (33.3)	0.676
Ile/Val	62 (48.1)	19 (52.8)	
Val/Val	14 (10.9)	5 (13.9)	
Ile	0.65	0.60	
Val	0.35	0.40	0.399
MnSOD, *N* (%)			
Ala/Ala	30 (23.3)	10 (27.8)	0.359
Ala/Val	71 (55.0)	22 (61.1)	
Val/Val	28 (21.7)	4 (11.1)	
Ala	0.51	0.58	0.256
Val	0.49	0.42	

higher expression of these antioxidant enzymes, increased beta-cell ROS scavenging and improved beta-cell survival after treatment with different sources of ROS (34). Although the functional implications are attractive, their relevance is not reflected by the data from our case–control association study.

The lack of association between GSTP1 and MnSOD genotypes, and CAP confirms data from another recent report in which patients with CAP were included (25). In this relatively small analysis, only 75 patients with CAP were included in addition to 33 patients with idiopathic chronic pancreatitis and 13 individuals with hereditary pancreatitis. No association was found for variants of both genes, but a significantly higher frequency of genotype GSTT1*A was detected in patients with idiopathic chronic pancreatitis. However, this association seems largely underpowered with a subgroup size of 33 patients, and is likely attributable to a type-I error which refers to false-positive associations frequently seen in studies with a low sample size. On the other hand, our data contrast with another study from Brazil that reported an association of genotype GSTP1 Val/Val with chronic pancreatitis in alcoholics, but only 14 patients with pancreatitis were analyzed. Again, such a small sample size is too small to draw firm conclusions.

Although the pathophysiology of acute and chronic pancreatitis are distinct, interestingly, our data are in line with a previous study by Rahman *et al*. (22) who investigated the role of polymorphisms of a battery of antioxidant enzymes including GSTM1, T1, and P1, manganese superoxide dismutase (Ala-9Val) and catalase (c-260T) in the development of acute pancreatitis, a disease somehow distinct from chronic pancreatitis. The authors genotyped 320 patients with acute pancreatitis due to alcohol consumption (n = 66), biliary diseases (n = 194) or other causes (n = 60), as well as 263 control subjects. They found an association between the functional GSTT-1*A genotype and severe versus mild acute pancreatitis. This genotype was also associated with higher C-reactive protein levels and acute physiology scores. However, this association only held true for acute pancreatitis due to biliary causes, but not for that related to alcohol consumption.

As regards GSTP1 genotypes and the development of CAP, Verlaan *et al*. (23) made similar observations, however, in a much smaller number of patients. In their study, the GSTM1-null genotype was found to be a protective factor towards the evolution of chronic pancreatitis due to alcohol, but not towards other etiologies.

Apart from demonstrating the lack of association between the two tested genes and CAP, our study illustrates the need to apply stringent quality criteria when performing candidate gene association studies. The controversy between strong associations of candidate genes and certain disease end points in initial reports and the inability to reproduce these data in subsequent studies is mostly due to the fact that results of ‘index’ studies were obtained in small case–control studies with an incomplete characterization of cases and controls. Often, no statistical correction for potential confounding factors, such as alcohol consumption, cigarette smoking and age, was performed and many other pivotal aspects in designing this kind of study were not adopted. In this regard, several prerequisites for the design

of genotype/phenotype association studies are currently considered crucial (35–37): (i) there should be a logical rationale for the chosen candidate genes and a coherent hypothesis based on the functional significance of the studied genetic variants; (ii) in case–control studies, several functionally related genes should be tested since this approach has a higher chance to detect genetic risk factors than the screening of single genetic variants; (iii) inclusion of well-characterized cases and controls ensuring a comparable exposure to the pathogenic insult (i.e. alcohol); (iv) a study sample with a homogeneous ethnic genetic background; (v) control for or exclusion of other confounding factors and (vi) application of accepted standards for statistical analysis and sample size, especially with regard to the size of the group of 'cases' (i.e. CAP). As regards the latter, a sample size of $\geq$150 in the 'case' group has recently been defined as a critical threshold for the replication validity of genetic association studies (38). All these demands were considered in the present study. Therefore, in summary, our data indicate that GSTP1 and MnSOD genotypes can be firmly considered irrelevant in the development of CAP as well as their complications exocrine and endocrine pancreatic insufficiency.

Acknowledgements

F.S. received a research fellowship from the *Interdisciplinary Centre of Clinical Research of the University of Erlangen-Nuremberg (IZKF)* and original research is funded by grants from the Novartis Foundation and the Liver Foundation, Berne, Switzerland. C.H.Ö. is supported by an *Erwin Schroedinger* research fellowship kindly provided by the *Austrian Science Fund (FWF)*.

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Received on February 19, 2007; revised on March 30, 2007; accepted on April 3, 2007